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Identification of Subunits *a*, *b*, and c_1 from *Acetobacterium woodii* Na^+-F_1F_0-ATPase

SUBUNITS c_1, c_2, AND c_3 CONSTITUTE A MIXED *c*-OLIGOMER*

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The Na^+-F_1F_0-ATPase operon of *Acetobacterium woodii* was recently shown to contain, among eleven *atp* genes, those genes that encode subunit *a* and *b*, a gene encoding a 16-kDa proteolipid (subunit c_1), and two genes encoding 8-kDa proteolipids (subunits c_2 and c_3). Because subunits *a*, *b*, and c_1 were not found in previous enzyme preparations, we re-determined the subunit composition of the enzyme. The genes were overproduced, and specific antibodies were raised. Western blots revealed that subunits *a*, *b*, and c_1 are produced and localized in the cytoplasmic membrane. Membrane protein complexes were solubilized by dodecylmaltoside and separated by blue native-polyacrylamide gel electrophoresis, and the ATPase subunits were resolved by SDS-polyacrylamide gel electrophoresis. N-terminal sequence analyses revealed the presence of subunits *a*, c_2, c_3, *b*, δ, α, γ, β, and ε. Biochemical and immunological analyses revealed that subunits c_1, c_2, and c_3 are all part of the *c*-oligomer, the first of a F_1F_0-ATPase that contains 8- and 16-kDa proteolipids.

Acetobacterium woodii is a strictly anaerobic, homoacetogenic bacterium that relies on a sodium ion potential across its cytoplasmic membrane for energy-dependent reactions (1). The sodium ion potential is established by a not yet identified primary pump connected to the acetyl-CoA pathway (2, 3). The transmembrane electrochemical Na^+ gradient established is used as the driving force for flagellar rotation as well as ATP synthesis (3–5). The enzyme catalyzing Na^+-driven ATP synthesis was purified and characterized by immunological methods, inhibitor studies, and molecular analyses as a Na^+-F_1F_0-ATPase (6–12).

The *atp* operon encoding the Na^+-F_1F_0-ATPase of *A. woodii* was recently cloned, sequenced, and shown to consist of the genes *atpI*, *atpB*, $atpE_1$, $atpE_2$, $atpE_3$, *atpF*, *atpH*, *atpA*, *atpG*, *atpD*, and *atpC*. The finding of multiple copies of genes ($atpE_1$, $atpE_2$, $atpE_3$) encoding subunit *c* homologues (the so called proteolipids) is without precedence in any bacterial species. Subunits $AtpE_2$ (c_2) and $AtpE_3$ (c_3) are identical on the amino acid level. Their deduced molecular mass is 8.18 kDa, and they are predicted to be organized in the membrane as a hair pin connected by a polar loop (13). Each hair pin contains an ion-binding site (10, 14, 15). $AtpE_1$ (c_1) most likely arose by duplication of an ancestral gene and subsequent fusion of the gene copies. Subunit c_1 is predicted to have four transmembrane helices, but the ion binding motif is conserved only in hair pin one, but not two. Therefore, subunit c_1 of *A. woodii* is similar to the so-called 16-kDa proteolipids of V_1V_0-ATPases, which also arose by gene duplication accompanied by loss of the proton-binding residue in hair pin one. The loss of the proton-binding residue was believed to be the reason for the apparent inability of V_1V_0-ATPases to function as ATP synthases under *in vivo* conditions (16). Western blot analyses verified that $atpE_1$ was expressed and that the product was not posttranslationally split into two 8-kDa proteolipids (11). However, $AtpE_1$ was not found in the enzyme purified previously (6). The Na^+-F_1F_0-ATPase of *A. woodii* purified previously not only lacked subunit c_1 but also the gene products AtpB (subunit *a*) and AtpF (subunit *b*). Subunits *a* and *b* were also not present in the ATPase of *Moorella thermoacetica*, although the encoding genes were present. These findings led to the hypothesis that *atpB* and *atpF* are transcribed, but the messages are not translated (17, 18). The finding of the genes $atpE_1$, *atpB*, and *atpF* in the F_1F_0-ATPase operon of *A. woodii* raised the question whether subunits c_1, *a*, and *b* are true subunits of the F_1F_0-ATP synthase of *A. woodii*. We demonstrate here that the genes encoding subunits *a* and *b* of *A. woodii* are expressed and that subunits *a*, *b*, and c_1 are assembled into the ATPase complex. This is the first demonstration of an F_1F_0-ATPase containing a heterooligomer of subunit *c* consisting of both 8- and 16-kDa proteolipids.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were reagent grade and purchased from Merck AG (Darmstadt, Germany). Antibodies were prepared by Bioscience (Göttingen, Germany).

Organisms and Plasmids—A. woodii (DSMZ 1030) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) and grown under strictly anaerobic conditions on carbonate-buffered medium supplemented with 0.4% glycine (19). *Escherichia coli* DH5α (*supE*44 Δ*lac*U169 (Φ80*lacZ*ΔM15) *hsdr*17 *recA*1 *endA*1 *gyrA*96 *thi*1 *relA*1) (20) was grown on luria broth (LB) at 37 °C. Plasmid pMalc2X was purchased from New England Biolabs.

Expression of atpB, atpE1, atpF, and atpD in E. coli and Generation of Antibodies—Base pairs 433–663 of *atpB* (named *atpB**) were amplified by polymerase chain reaction using oligonucleotides $PatpB_1$ (5′-GTAATTGGGGAATTCGCTAATCCC-3′) and $PatpB_2$ (5′-GTTCCCTCCAAGCTGCAGCATAA-3′). Base pairs 190–552 of *atpF* (named atpF*) were amplified using $PatpF_2$ (5′-CGTATTTACCTGCAGCTAAACTCA-3′) and $PatpF_3$ (5′-GTGACGGCTGAATTCCTCGG-3′). *atpD* was amplified using primers $PatpD_1$ (5′-GGTTAGTGGAATTCGCCC-3′) and $PatpD_2$ (5′-TCTGAAAGCTGCAGCCATTA-3′). The polymerase chain reaction fragments were cloned into pMalc2X, and the plasmids were transformed in *E. coli* DH5α. Cultures were grown in LB at 37 °C, and expression was induced at an A_{600} of 0.5 by addition of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. After 2 h of growth, cells were harvested,

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washed, and disrupted at high pressure in a French press. Because there is no MalE in *A. woodii* and because a MalE antibody does not cross-react with cell-free extract of *A. woodii*, the entire fusion protein was used to immunize rabbits.

Chloroform/Methanol Extraction of A. woodii Membranes—Membranes were prepared as described previously (6). Chloroform/methanol extraction of *A. woodii* membranes was performed as described (21) with 160 mg of membrane protein dissolved in 8 ml of 50 mM Tris, pH 8. The extracts were precipitated twice with four volumes of diethylether as described in Ref. 22. The 8-kDa proteolipid was electroeluted from an SDS-polyacrylamide gel and used for immunization of a rabbit.

Immunoblotting—SDS-PAGE[1] and Western blotting were performed as described previously (11, 23). Transfer of proteins from blue native-PAGE to polyvinylidene difluoride membranes was essentially as described (24).

Blue Native-PAGE—Washed membranes were first pelleted by centrifugation at 140,000 × *g* for 1 h and resuspended in 50 mM imidazole (pH 7.0), 50 mM NaCl, 2 mM aminocaproic acid, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Membrane proteins were then solubilized with dodecylmaltoside (1 g/g of protein) for 20 min on ice. Thereafter, membranes were pelleted by centrifugation at 140,000 × *g* for 30 min. The supernatant was subjected to blue native-PAGE as described (24), except that the cathode buffer contained 7.5 mM imidazole (pH 7.0), 50 mM tricine, and 0.02% Serva Blue and the anode buffer contained 50 mM imidazole (pH 7.0).

RESULTS

Expression of atpB, atpE$_1$, atpF, and atpD in E. coli—To generate antibodies against subunits *a*, *b*, and c_1, the genes *atpB*, *atpF*, and *atpE*$_1$ of *A. woodii* were fused to *malE* and expressed in *E. coli*, and the fusion proteins were used to immunize rabbits. Because attempts to express full-length *a*, *b*, and c_1 fusions in *E. coli* were unsuccessful, deletion derivatives were made. From *atpB* the 3′-terminal 230 base pairs were fused to *malE*. Expression was low in this case, but after purification the quantity of MalE-AtpB* was sufficient for immunization of rabbits. A *malE-atpD* fusion gave high expression yields. In case of *atpE*$_1$ a sequence of 66 base pairs, coding for the first hydrophilic loop of subunit c_1, was fused to *malE*. This sequence was chosen to minimize cross-reactions of the antiserum with subunit $c_{2/3}$, because only 10 of 22 amino acids in this sequence are identical in subunit c_1 and $c_{2/3}$. This construct was expressed in appreciable amounts. 362 base pairs of *atpF*, coding for a part of the hydrophilic domain, were fused to *malE*, and the fusion gene was also expressed in sufficient amounts.

Immunological Detection of Subunits a and b in the Cytoplasmic Membrane of A. woodii—*A. woodii* was grown on 20 mM fructose to an A_{600} of 0.8 (logarithmic growth phase) and harvested, and cytoplasmic and membrane fractions were prepared. After SDS-PAGE, the proteins were blotted on nitrocellulose membranes and probed with different polyclonal antisera.

The antiserum against subunit β reacted, as expected, with a protein having an apparent molecular mass of 51 kDa (Fig. 1), which is identical to the deduced molecular mass of subunit β. Subunit β was found predominantly in the membrane fraction but also in the cytoplasm. The strongest reaction of the antiserum against subunit *a* was with a 29-kDa membrane protein (Fig. 1). At higher protein concentrations (>25 μg) 18- and 15-kDa membrane proteins also reacted with the anti-subunit *a* antiserum. The deduced molecular mass of subunit *a* is 24.5 kDa. Because subunit *a* from *E. coli* and *Propionigenium modestum* migrates in SDS-PAGE at molecular masses lower than expected from the deduced sequence (25, 26), it is unlikely that the predominant 29-kDa signal corresponds to subunit *a*. Concomitantly, the N-terminal sequence analyses

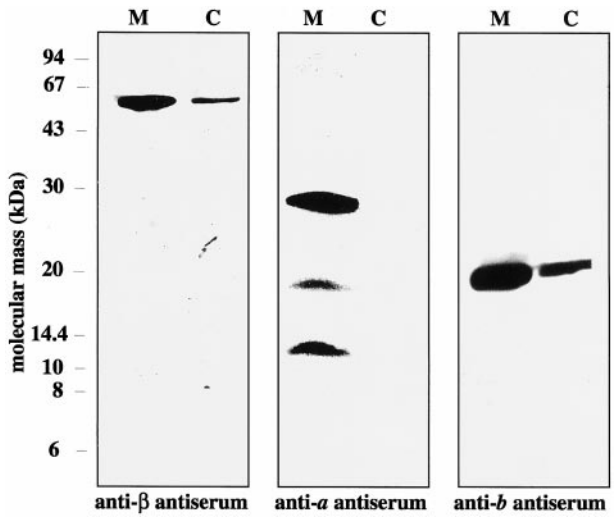

FIG. 1. **Western blot analyses of *A. woodii* membranes and cytoplasm with antisera against subunits β, *b*, and *a*.** SDS-PAGE (10%) was performed with 10 μg of membrane (*M*) and cytoplasmic (*C*) protein to detect subunits β and *b*. For detection of subunit *a* the gels were loaded with 50 μg of protein in each lane.

presented revealed that the 18-kDa protein is subunit *a*. The antiserum raised against subunit *b* reacted with a protein having an apparent molecular mass of 19 kDa, which is quite similar to the deduced molecular mass of 20.8 kDa. Subunit *b* was also found predominantly in the cytoplasmic membrane. These experiments demonstrate that subunits *a* and *b* are produced and located in the cytoplasmic membrane of *A. woodii*.

Specificity of the Antisera against c_1 and $c_{2/3}$—For further studies it was important to clearly establish the specificity of the antisera generated against the different proteolipids. The antiserum against subunit c_1 reacted with subunit c_1 (apparent molecular mass of 16 kDa) in membranes of *A. woodii*, as observed before, but in addition a band at 43 kDa was obtained (Fig. 2). This band represents the *c*-oligomer, as determined by N-terminal sequencing (see below). Apparently, the anti-c_1 antiserum does not cross-react with subunits $c_{2/3}$. The anti-$c_{2/3}$ antiserum reacted with subunits $c_{2/3}$ and the *c*-oligomer but not with subunit c_1. Only at very high protein concentrations (>100 μg) was there a weak cross-reaction of the anti-$c_{2/3}$ antiserum with c_1 (data not shown). These results show that subunits c_1 and $c_{2/3}$ are present in membranes of *A. woodii* in monomeric and oligomeric forms. In silver-stained SDS-polyacrylamide gels of chloroform/methanol extracts, two major proteins having apparent molecular masses of 16 and 7 kDa were observed. In Western blots, the anti-$c_{2/3}$ antiserum reacted with the 7-kDa polypeptide, which was identified as subunit $c_{2/3}$ by N-terminal sequencing, and to a much lesser extent with the 16-kDa polypeptide. The anti-c_1 antiserum reacted only with the 16-kDa polypeptide (Fig. 3). These studies verified that the anti-c_1 antiserum does not react with subunit $c_{2/3}$. The reaction of the anti-$c_{2/3}$ antiserum with subunit c_1 is expected, because 60 and 72% of the amino acids of subunits $c_{2/3}$ are conserved in the first and second half of subunit c_1, respectively (11).

Subunit Composition of the Native ATPase—Membrane proteins were solubilized with Triton X-100, dodecylmaltoside, and laurylmaltoside in different concentrations, ranging from 1 to 24 g of detergent/g of protein. The solubilized protein complexes were then separated by blue native-PAGE. Independent of the nature and concentration of the detergent used, a predominant protein band with an apparent molecular mass of 590 kDa was observed (Fig. 4). In addition, bands with much lower intensi-

[1] The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

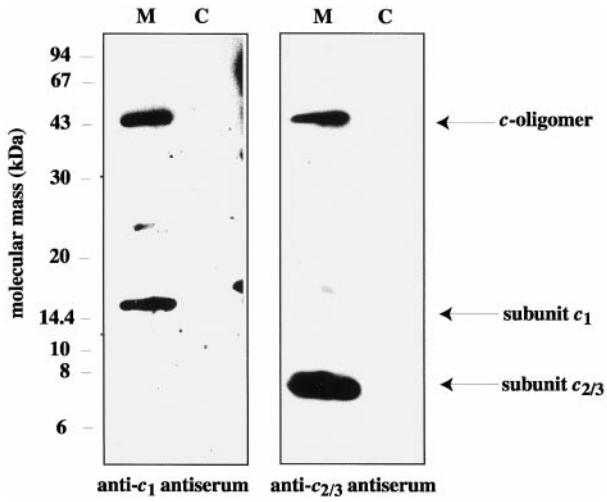

FIG. 2. **Western blot analyses of *A. woodii* membranes and cytoplasm probed with anti-c_1 and anti-$c_{2/3}$ antisera.** SDS-PAGE (10%) was performed with 10 μg of cytoplasmic (*C*) and membrane (*M*) protein per lane for anti-$c_{2/3}$ antiserum and 50 μg per lane for anti-c_1 antiserum.

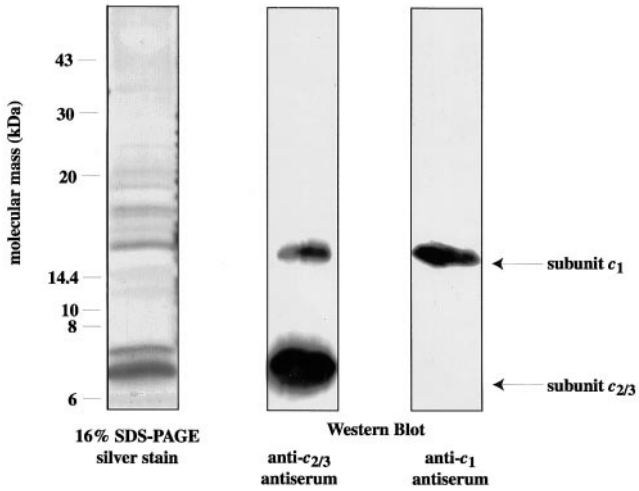

FIG. 3. **Substrate specificity of anti-c_1 and anti-$c_{2/3}$ antisera on chloroform/methanol extracts.** Membranes of *A. woodii* were extracted with chloroform/methanol, and proteins were precipitated with diethylether and loaded on a 16% SDS polyacrylamide gel. Subunit $c_{2/3}$ was identified by N-terminal sequencing.

ties were observed at 300 and 150 kDa. The apparent molecular mass of the 590-kDa complex corresponds well to the molecular mass of the F_1F_0-ATPase from *A. woodii*. That this complex indeed represents the ATPase was verified by Western blot analyses using the anti-β antiserum as probe (data not shown).

When the membrane protein complexes were separated in the first dimension by blue native-PAGE and in the second dimension by SDS-PAGE, the subunits of the ATPase complex were resolved (Fig. 4). Eight polypeptides with apparent molecular masses of 58, 55, 43, 37, 21, 19, 18, and 16.5 kDa were detected by silver staining. N-terminal sequencing of these proteins gave clear evidence that they are subunits α and β, the *c*-oligomer, and subunits γ, δ, *b*, *a*, and ϵ, respectively. This experiment gives clear evidence that subunit *a* and *b* are present in the enzyme complex. However, c_1 and $c_{2/3}$ monomers could not be detected; only the *c*-oligomer could be detected. N-terminal sequencing of the polypeptides in the oligomer clearly revealed the presence of $c_{2/3}$, but c_1 was not detected. To detect c_1 in the complex, an immunological approach was chosen.

Immunological Detection of c_1 in the Native Enzyme—To identify subunit c_1 in the native ATPase, the ATPase complex was resolved by blue native-PAGE and SDS-PAGE as described above, blotted on nitrocellulose membranes, and probed with anti-c_1 and anti-$c_{2/3}$ antiserum. Both antisera reacted with the *c*-oligomer but not with monomeric subunits c_1 and $c_{2/3}$ (Fig. 5). Nevertheless, this proves that subunit c_1, besides subunit $c_{2/3}$, is present in the *c*-oligomer. The *c*-oligomer of the *A. woodii* ATPase can be disrupted by autoclaving it at 120 °C for 3 min (6). As can be seen from Fig. 5, this treatment leads to the disruption of the *c*-oligomer. Concomitantly, two polypeptides of 7 and 16 kDa appeared. The 7-kDa polypeptide was identified both immunologically and by N-terminal sequencing as subunit $c_{2/3}$. Unfortunately, the concentration of the 16-kDa polypeptide was too low for N-terminal sequencing, but the Western blot analyses clearly identified it as subunit c_1. Taken together, these experiments gave clear evidence that c_1 is assembled into the ATPase complex and is part of the *c*-oligomer. This demonstrates, for the first time, the presence of a duplicated proteolipid in an F_1F_0-ATPase.

DISCUSSION

We have now isolated the Na^+-F_1F_0-ATPase in its native state and found nine polypeptides. These were identified by N-terminal sequencing and immunological methods as subunits *a*, c_1, $c_{2/3}$, *b*, δ, α, γ, β, and ϵ. The N-terminal sequences now available allow us to identify unequivocally the start codons of the respective genes. With the exception of *atpF*, the experimentally determined start codons match the ones deduced from the DNA sequences. The start codon of *atpF* is actually 45 nucleotides downstream from the previously assumed start site (12). Translation of *atpF* starts with the unusual start codon TTG; the same is true for *atpA* (11). *N*-formylated N-terminal methionines were found in subunits *a*, *b*, and $c_{2/3}$, whereas subunit α has a deformylated methionine. The N-terminal methionine was removed from subunits β, γ, δ, and ϵ. Removal of the first methionine was also reported for subunits β, γ, and ϵ of the *E. coli* enzyme (27) and for subunits γ and ϵ of the *P. modestum* enzyme (26).

ATPase preparations from *A. woodii* described previously lacked subunits *a* and *b*. The same was observed in *M. thermoacetica* and *Moorella thermoautotrophica*, although the encoding genes were present in the *atp* operons (17, 18). *atpB* and *atpF* of *M. thermoacetica* were transcribed, but because antisera against synthetic polypeptides derived from the sequences of subunit *a* and *b* of *M. thermoacetica* did not cross-react with cell free extract (18), it was concluded that the messages are not translated. From the findings presented here it is clear that in *A. woodii* subunits *a* and *b* are produced. By using the gentle blue native-PAGE procedure, we were able to isolate the ATPase complex in its native state. Therefore, we have to conclude that subunits *a* and *b* were lost in the course of the purification procedure employed in a previous study. Even with the use of blue native-PAGE, subunit *a* was not detectable in every preparation.

Another striking and unique feature of the Na^+-F_1F_0-ATPase of *A. woodii* is its duplicated proteolipid, subunit c_1. This is without precedence in bacteria. Duplicated proteolipids were, for a long time, seen as an exclusive feature of eucaryal V_1V_0-ATPases (28). In archaea, duplication and triplication of proteolipid-encoding genes with subsequent fusion of the genes was described very recently (16, 29). With the experiments described here we add another argument, now derived from a bacterial species, that multiplied and fused proteolipid-encoding genes are not exclusively present in eucarya, but also in the other domains of life. From the experiments described here it is clear that subunits $c_{2/3}$ and c_1 constitute the *c*-oligomer. Although the stoichiometry of the individual polypeptides of the

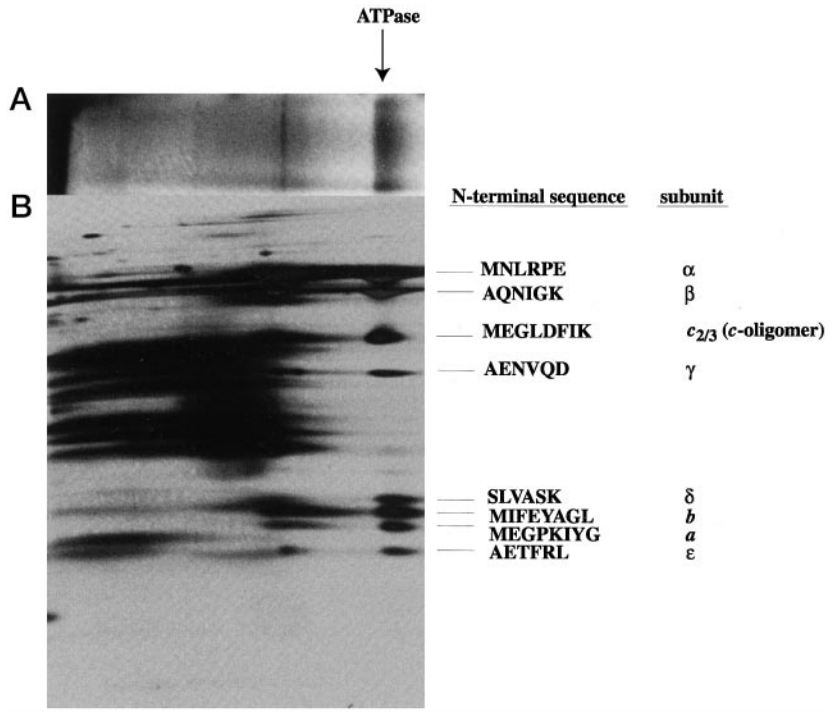

FIG. 4. **Isolation of ATPase by blue native-PAGE and identification of the ATPase subunits by N-terminal sequencing.** Membrane protein complexes were separated in the first dimension by blue native-PAGE (*A*) and in the second dimension by SDS-PAGE (10%) (*B*). The gel was blotted on polyvinylidene difluoride membrane, and the N termini of the ATPase subunits were determined by Edman degradation.

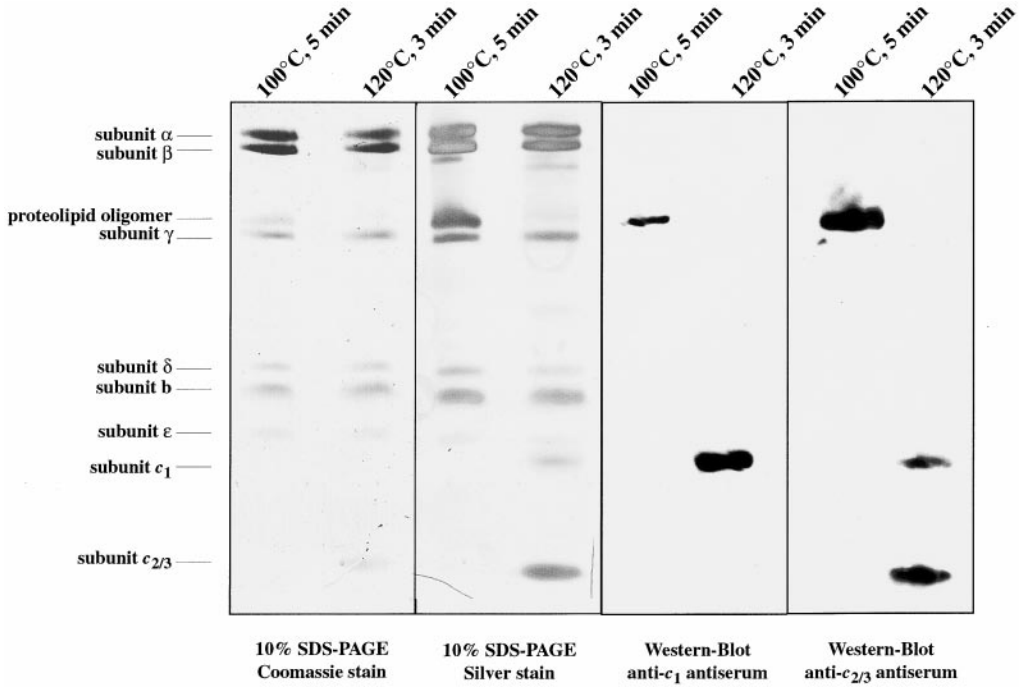

FIG. 5. **Immunological detection of subunit c_1 in the *c*-oligomer.** The ATPase complex was cut out of the blue native-PAGE, denatured as indicated, separated by SDS-PAGE, and stained as indicated. The polyacrylamide gels were blotted onto nitrocellulose membranes and probed with anti-c_1 or anti-$c_{2/3}$ antisera. Subunit *a* was not present in this preparation or not separated from subunit *b*.

oligomer is unknown, it appears from the Western blots and SDS-PAGEs that subunit c_1 is only a minor component. In this connection it should be mentioned that the migration behavior of the *c*-oligomer is dependent on the acrylamide concentration. In 10% SDS-polyacrylamide gels, the *c*-oligomer runs at 43 kDa, but in 16% gels, it runs at 61 kDa. Therefore, we cannot speculate about the number of monomers in the complex.

Subunit c_1 is not only duplicated, but Glu-162 in hair pin two is also substituted by a glutamine residue. The glutamate is part of the proposed sodium ion-binding site (Pro-Gln-Glu-Thr) (10, 15, 30) in subunit *c*. Although the free electron pair of the amino group of Gln-162 could in principal bind the sodium ion (as does Gln-46 in helix one and Gln-129 in helix three), the substitution might have consequences for the rotation of the motor of the ATPase. Current views on the function of the motor assume an electrostatic attraction of Na^+ (H^+) by Glu (Asp) (31, 32). Due to the neutralization of the charge of Glu (Glu-62 in $c_{2/3}$ and Glu-79 in c_1) after coordinating a sodium ion, the *c*-ring may cross the electric barrier and rotate into the hydrophobic zone, driven by the electrostatic interaction of a highly conserved Arg (Arg-158 in *A. woodii*) in subunit *a* with another free Glu on the next monomer of the *c*-ring. This would lead to a rotation of the *c*-ring relative to subunits *a* and *b*. The lesser the number of carboxylates per ring, the worse is the

coupling efficiency. In the worst case, the V_1V_0-ATPases, ATP synthesis (under physiological conditions) is abolished, but proton pumping capacity is increased. For the F_1F_0-ATPase of *E. coli* it was demonstrated that the ATPase can tolerate the exchange of one Asp-61 with an Asn residue without losing its capability to translocate H^+ (33). The ATPase from *Methanococcus jannaschii* contains a triplicated proteolipid with only two proton-translocating groups, but this enzyme still functions as an ATP synthase. In view of this discussion, the determination of the exact stoichiometry of the subunits of the *c*-oligomer of *A. woodii* is essential; this remains a challenging task for the future.

What could be the function of the two different proteolipids in the ATPase of *A. woodii*? Although it is hard to speculate at present, an attractive idea is the regulation of the function of the enzyme by the relative stoichiometry of $c_{2/3}$ and c_1. As pointed out above, the higher the ratio of $c_{2/3}$ over c_1, the better the enzyme functions as an ATP synthase, whereas a high c_1:$c_{2/3}$ ratio favors pump activity. During growth on fermentable substrates such as sugars, the enzyme may function as an ATP-driven ion pump used to regulate intracellular pH and/or Na^+ concentration, whereas during growth on H_2 + CO_2 the enzyme has to drive ATP synthesis by means of the electrochemical Na^+ potential across the membrane. Regulation of the coupling efficiency in ATPases by varying the number of *c*-subunits per oligomer was originally suggested by Brusilow and co-workers (34). Verification of this interesting idea remains a challenging task for future experiments.

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